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We report progress on the use "DNA nanocircle vectors" to encode the synthesis of ribozymes in living cells. Ribozyme-encoding circular DNA randomized libraries were used to select for strongly transcribed sequences with specific RNA polymerases. We identified new ssDNA promoters for bacterial enzymes and built new DNA nanocircles encoding ribozymes. These were demonstrated to be biologically active in bacterial cells. A similar strategy may one day be useful in the future for inhibiting genes (such as breast cancer-associated genes) in human cells.

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GRANT # DAMD17-98-1-8239

TITLE: **Rolling Circle Transcription of Ribozymes Targeted to *ras* and mdr-1**

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INTRODUCTION

This IDEA project focuses on a new method for generating biologically active ribozymes (1-11). This method is termed "rolling circle transcription", and involves unusual circular single-stranded DNA templates. We have previously discovered that synthetic DNA circles as small as ~30 nucleotides in length could be transcribed efficiently by RNA polymerases, despite their lack of promoter sequences. When we encode ribozymes in these circles, as well as their self-cleavage sequences, then this produces a long string of ribozymes that self-cleaves until virtually the only product is a ribozyme (in amplified quantities) that has the same length as the circle (12-15).

We proposed the interesting possibility that such circular DNAs might one day be used in cells to encode biologically active ribozymes. These ribozymes might target disease-related RNAs for destruction, and thus the circular DNAs could have a biological effect. In breast cancer, two mRNA targets that are likely to be important are H-ras and mdr-1. H-ras is an oncogene that is commonly mutated and overexpressed in breast cancer; it is possible that downregulation of this mutated gene would affect cancer growth in a favorable way. The gene mdr-1 is very commonly overexpressed in malignancies that Have undergone anticancer therapy; this leads to drug resistance in further treatment. Thus, downregulation of mdr-1 RNA might well lead to an enhanced ability to treat breast cancer with standard drugs.

Before our synthetic circular DNAs can be used as vectors in breast cancer therapy, quite a number of questions need to be answered, and some of these are the subject of this ongoing project. We need to measure the RNA-cleaving activity of ribozymes produced by rolling circle transcription. Are they as efficient as standard ribozymes? We also need to find sequences of DNA circles that are most efficiently transcribed. Can transcription be made more efficient than we have already observed? We need to find

sequences that lend optimal activity and stability in cellular media. Finally, we would like to optimize transcription by cellular polymerases. Can these more complex polymerases utilize such small circles as templates?

Below is an outline of the proposed statement of work for this project:

Task 1. Compare cleavage activity and nuclease stability of self-processed vs. conventional ribozymes

Task 2. Optimize in vitro transcription and investigate incorporation of modified ribonucleotides

Task 3. Evaluate the most active and stable RNAs in cell culture

Task 4. Carry out in vitro selection to optimize transcription of such vectors by human RNA polymerases

In the third year of this project we made significant progress on all proposed tasks, with strongest progress in tasks 2-4, having completed task 1 as planned. Below are described details of our work on the remaining three tasks.

TASK 1: Cleavage activity of self-processed ribozymes:

--completed in year 1--

TASK 2: Optimizing in vitro transcription:

--completed in year 2—

Over the past two years we completed an optimization of transcription by two different classes of polymerases, one a viral RNA polymerase (T7 RNA polymerase), and one a bacterial polymerase (E. coli RNA polymerase). A 103-nt circular DNA was prepared, encoding the 63-nt mdr-1 ribozyme as well as a 40 nt randomized domain. The method used to construct the molecule was as done before, using two shorter linear DNAs and ligating them in two steps. This yields a circular DNA library of ca. 10^{13} different sequences.

This library was then used in an in vitro selection scheme, in which we select for sequences that are transcribed and yield monomer ribozyme RNAs. The scheme briefly works as follows: the library is transcribed and the RNAs are allowed to self-process. They are separated on a gel next to a size marker. The monomer-length RNAs are excised from the gel. They are amplified by RT-PCR and then the DNAs are re-cyclized. This new set of circular DNAs has thus been enriched in sequences that are especially well transcribed. Moreover, the corresponding RNAs had to retain high self-cleavage activity to appear as the monomer band on the gel. Additional rounds lead to further enrichment. At the end of the selection, the PCR fragments are cloned and sequenced. This yields optimized circle sequences, all of which encode the mdr-1 ribozyme.

Materials and Methods

Preparation of Oligonucleotides and Circular DNA Library. DNA oligonucleotides were synthesized on solid supports using the phosphoramidite method on an Applied Biosystems model 392 DNA/RNA synthesizer. Oligodeoxyribonucleotides were deprotected by treatment with concentrated ammonia at 55°C for 8 h. After deblocking, DNA oligonucleotides were purified by electrophoresis on polyacrylamide denaturing gels (PAGE). After elution from the gels, the oligonucleotides were desalted on C18 Sep-Pak cartridges. Single-strand concentrations of purified DNA oligonucleotides were determined by measuring the absorbance at 260 nm at high temperature. Single-strand extinction coefficients were calculated from mononucleotide and dinucleotide data with a nearest-neighbor approximation (1).

An initial circular single-stranded DNA library containing 63 nt fixed sequence and 40 nt randomized sequence was generated by sequential enzymatic ligations of 5'-phosphorylated 56 nt and 47 nt oligonucleotides using T4 DNA ligase (New England Biolabs) and 16 nt splint oligonucleotides as described (2). The 5'-phosphorylated sequences were: 5'-pTTC GTC TG-N_{40}-TCT TTC AG-3' and 5'-pTTT CGT CCT CAC GGA CTC ATC AGA ATG GCA ACA CAT TGA CTG AGG AG-3'.

***In vitro* selection.** Conditions for initial rolling circle transcription reaction were: 1 μM circular DNA, 2 units *E. coli* RNA polymerase holoenzyme (Boehringer Mannheim), 0.5 mM ATP, CTP, and GTP, 60 μM UTP, and 0.30 μCi (1 Ci = 37GBq) of [α-^{32}P]UTP in 25 mM Tris•HCl (pH 8.1) buffer containing 20 mM NaCl, 15 mM $MgCl_2$, 0.4 mM spermine•HCl, 100 μg/mL acetylated bovine serum albumin, 10 mM dithiothreitol, and 12.5 units/mL RNase inhibitor (Promega), in a total reaction volume of 15 μL. After 1.5 h incubation at 37°C, the reaction was terminated by adding an equal volume of stop solution (30 mM Na_2EDTA/8 M urea/0.02% bromophenol blue/0.02% xylene cyanol). Self-processed 103-nt product RNAs were purified by 10% denaturing PAGE gels run at 4°C.

After elution from the gels, the selected 103-nt ssRNAs were reverse transcribed (AMV Reverse Transcriptase, Invitrogen) with a 5'-phosphorylated 18-nt primer (5'-pGAC TGA GGA GTT CGT CTG-3') for 1 h at 42°C. After buffer exchange with Bio-spin column (Bio-Rad), the cDNA products were PCR amplified with *Taq* polymerase for 15 cycles (temperature cycle: 94°C, 1 min; 55°C, 1 min; 72°C, 1 min) in the presence of 100 pmol each of two primers: 5'-pGAC TGA GGA GTT CGT CTG-3' and 5'-biotin-AAT GTG TTG CCA TTC TGA-3'. The PCR products were extracted with phenol/$CHCl_3$, ethanol precipitated, and blunted with T4 DNA polymerase (LifeTechnologies, Grand Island, NY).

The blunt-ended PCR products were immobilized on magnetic beads with streptavidin (Dynal) in the presence of 80 μM binding buffer [1 M NaCl/10mM Tris•HCl (pH7.5)/1mM EDTA], rinsed with two 80 μL volumes of the binding-buffer, and eluted with a 70-μL volume of 0.15 N NaOH to recover the nonbiotinylated ssDNA. The recovered solution was then ion exchanged with a spin-column (Bio-Spin P-30 Column, Bio-Rad).

After quantifying the nonbiotinylated ssDNA with 5'-phosphate by measuring the absorbance at 260 nm, 1 μM nonbiotinylated ssDNA with 5'-phosphate was mixed with 2 μM 24 nt splint DNA (5'-AAC TCC TCA GTC AAT GTG TTG CCA-3') in the presence of 10 mM Tris•HCl (pH 7.5) and 10 mM $MgCl_2$, annealed with a thermal cycler (rate, 1°C/min) from 90°C to 25°C, and incubated with 0.3 units of T4 DNA ligase, 0.1 mM dithiothreitol, and 10 mM ATP at 25°C for 12 h in a final total reaction volume of 15 μL. The circular ssDNA, unreacted ssDNA, and splint ssDNA were ethanol precipitated and used as templates to begin the next round of *in vitro* selection without purification. The rolling circle transcription reaction for next round was done under the conditions described above.

Control experiments were carried out as above but eliminating the ligation step. The population without ligation contained 1 μM nonbiotinylated ssDNA with 5'-phosphate and 2 μM 24 nt splint DNA to mimic the selections performed ligation.

Cloning and Sequencing. The polymerase chain reaction products of the fifteenth round pool were ligated into a TA cloning vector (Invitrogen) and cloned into *E. coli* TOP10F' (Invitrogen). Plasmid DNAs were isolated and sequenced using BigDye terminator cycle sequencing kit (PE Applied Biosystems).

CAT assay. The INVαF' *E. coli* strain (Invitrogen) was used. After cells were transfected with the CAT vector, cells were cultured in LB containing 50 μg/mL ampicillin until the absorbance at 600 nm reached 0.1. Then 1 μL cell solution with 0.1 absorbance was transferred into 100 μL LB solution with the circular DNA and incubated until the absorbance at 600 nm became 0.1-0.2. Cells were heat-shocked (42 °C) once each hour. The CAT activities were measured with [^{14}C] chloramphenicol (100 μCi/ml) and a CAT enzyme assay system (Promega). The efficiency of CAT protein extraction was checked by reference to ß-galactosidase activity: cells were cotransfected with pSV-ß-Galactosidase Control Vector (Promega) and then the chemiluminescent signal due to ß-galactosidase was determined with ß-galactosidase enzyme system (Promega).

TASK 3: Evaluate most active RNAs in cell culture:

This task has been completed for the selected circular DNA motifs described above. It is reported in full in the publication (see Appendix). Briefly, we succeeded in finding circular DNAs that act as vectors in bacterial cells. Once they enter the cells, they are transcribed by RNA polymerase enzymes in the cells, which produces large amounts of ribozymes. These ribozymes cleaved a disease-related RNA (a drug resistance gene) in the live cells.

Materials and Methods

Synthesis and Activity of Selected Circular Single Stranded DNAs. All circular ssDNAs were made by sequential enzymatic ligations as indicated above. Sequences were: E1, 5'-pGAC TGA GGA GTT CGT CTG GCA ACG AAT CAG ACT CTT TCG GTG ACA TTG CCC AGT TTA T-3' and 5'-pTCT TTC AGT TTC GTC CTC ACG GAC TCA TCA GAA TGG CAA CAC ATT-3'; E38, 5'-pGAC TGA GGA GTT CGT CTG GCC ACG ATC TGA ATA GTC GTT CAT CCT CAG CGG TAG CGA A-3' and 5'-pTCT TTC AGT TTC GTC CTC ACG GAC TCA TCA GAA TGG CAA CAC ATT-3'; E15, 5'-pGAC TGA GGA GTT CGT CTG GTA AAG TAT GTT GCT ACG ACT TCT TTA TTT ACC ACG ATG C-3' and 5'-pTCT TTC AGT TTC GTC CTC ACG GAC TCA TCA GAA TGG CAA CAC ATT-3'; *mdr*63, 5'-pGAC TGA GGA GTT CGT CTG TCT TTC AGT TTC GTC CT-3' and 5'-pCAC GGA CTC ATC AGA ATG GCA ACA CAT T-3'; *mar*A, 5'-GAC ACT GGA GTT CGT CTG GTA AAG TAT GTT GCT ACG ACT TCT TTA TTT ACC ACG ATG C-3' and 5'-pAGA AAG TGT TTC GTC CTC ACG GAC TCA TCA GAG AGC GTT CAC TCT-3'; inactive *mar*A, 5'-pGAC ACT GGA GTT CGT CTG GTA AAG TAT GTT GCT ACG ACT TCT TTA TTT ACC ACG ATG C-3' and 5'-pAGA AAG TGT GTC GTC CTC ACG GAC TCA TCA GAG AGC GTT CAC TC-3'; *mar*A library, 5'-pAGT TCG TCT G-N40-A GAA AGT GTT-3' and 5'-p TCG TCC TCA CGG ACT CAT CAG AGA GCG TTC ACT CTG ACA CTG G-3'; short *mar*A (63nt), 5'-pGAC ACT GGA GTT CGT CTG AGA AAG TGT TTC GTC CT-3' and 5'-pCAC GGA CTC ATC AGA GAG CGT TCA CTC T-3'.

All rolling circle transcription reactions were done under condition as indicated above using *E. coli* RNAP (Boehringer Mannheim) with 0.2 μM circular ssDNA templates. Reactions were incubated at 37°C, and were stopped by the addition of one volume of stop solution. Gel analysis was on a 10% PAGE run at 4°C. The transcribed and self-processed RNAs were quantified with a radioanalytical scanner (Molecular Dynamics Storm 860).

***Mar*A - CAT Chimeric Reporter Construct**. The chloramphenicol transferase (CAT) gene fragment was amplified from pKK232-8 (Amersham Pharmacia Biotech) by PCR with 29-nt and 27nt primers. Primer sequences were: 5'-AGG TCG ACT ATG GAG AAA AAA ATC ACT GG-3' and 5'-GGT ACC CAA AAG GCC ATC CGT CAG GAT-3'. After purification from an agarose gel, the PCR product was amplified again by PCR with 81-nt and 29-nt primers. The 81-mer primer had 51 nt from the *mar*A gene and a*Hind* III site. The 29-nt primer contained a *Kpn*I site. Primer sequences were: 5'-CCC AAG CTT GTC ACT GGA GAA AGT GTC AGA GCG TTC GGG TTA CTC CAA ATG GCA CCT GCA AAT GGA GAA AAA AAT CAC TGG-3' and 5'-GGG GTA CCC AAA AGG CCA TCC GTC AGG AT-3'. After purification from an agarose gel, this *mar*A-CAT fragment (1300 nt) was ligated into pUC19 (Amersham Pharmacia Biotech) at the *Hind*III site and *Kpn*I site with T4 DNA ligase. The ligated plasmids were cloned into INVαF' *E. coli* (Invitrogen) and selected by blue/white colony color.

Synthesis and activity of selected circular ssDNAs. All circular ssDNAs were made by sequential enzymatic ligations as indicated above. Sequences of all circular DNAs are given above.
All rolling circle transcription reactions were done under condition as indicated above using *E. coli* RNAP (Boehringer Mannheim) with 0.2 μM circular ssDNA templates. Reactions were incubated at 37°C, and were stopped by the addition of one volume of stop solution. Gel analysis was on a 10% PAGE run at 4°C. The transcribed and self-processed RNAs were quantified with a radioanalytical scanner (Molecular Dynamics Storm 860).

TASK 4: In vitro selection with human RNA polymerases:

This is the last remaining long-term goal for this project, as the other tasks have been nearly completed. Our strategy for finding circular DNA sequences that are strongly transcribed by human RNA polymerases is the same as was successful with bacterial enzymes. Although the bacterial enzyme selection showed very good progress, the experiments using human cellular extracts have not as yet. We believe this is due largely to the weak transcriptional activity present in the extract. Nonetheless we plan to carry out additional rounds of selection in the hope that there are rare winner sequences that will emerge at later rounds. If this is not successful, then we plan to repeat the experiments with a new circular DNA library having a larger randomized domain. We may also try the sequences that were successful in bacteria.

KEY RESEARCH ACCOMPLISHMENTS

• We have completed the identification and sequencing of new classes of single-stranded DNA "promoters" that are highly active in transcription by viral and bacterial enzymes.

• We have shown that nanocircle vectors incorporating such "promoters" can be transcribed in living bacterial cells to produce ribozyme RNAs.

• We have shown that such vectors can be used to strongly downregulate a drug resistance gene in living cells.

REPORTABLE OUTCOMES

One paper has appeared in print since the last report:

T. Ohmichi, A. Maki, E. T. Kool, Efficient bacterial transcription of DNA nanocircle vectors with optimized single-stranded promoters, *Proc. Natl. Acad. Sci. USA* **2002**, 99, 54-59.

CONCLUSIONS

(1) In vitro selection methodology has been successfully used to find specific DNA circle sequences that are much more highly efficient than average sequences in transcription.
(2) Nanocircle vectors can be successfully used to deliver RNAs into bacterial cells.
(3) Concatemeric self-cleaving ribozyme constructs can be highly active in downregulating gene expression.

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APPENDIX

Reprint of a recent publication supported by this grant.

Efficient bacterial transcription of DNA nanocircle vectors with optimized single-stranded promoters

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Communicated by John I. Brauman, Stanford University, Stanford, CA, November 2, 2001 (received for review July 10, 2001)

We describe experiments aimed at establishing whether circular single-stranded DNAs can form promoters for bacterial transcription from small folded motifs. *In vitro* selection experiments were carried out on circular 103-nt DNA libraries encoding 40-nt randomized sequences as well as self-processing hammerhead ribozymes. Rounds of rolling circle transcription, reverse transcription–PCR, and recyclization were carried out to optimize transcription efficiency. Sequences were identified that are 80-fold more actively transcribed than the initial library by *E. coli* RNA polymerase (RNAP). The selected motifs were found to be more active than canonical *E. coli* promoters in the same context. Experiments also demonstrated that a single-stranded pseudopromoter identified by this selection can be transplanted to other circular DNA contexts and retain transcriptional activity. Results suggest that the promoter is localized in a short (≈40 nt) hairpin, which is smaller than canonical *E. coli* promoters. To test whether this pseudopromoter was active in bacterial cells, a synthetic DNA nanocircle vector encoding a ribozyme targeted to a site in the *marA* drug resistance gene was constructed to contain an optimized single-stranded promoter. It is shown that this DNA circle can act as a "Trojan horse" in *E. coli*, being actively transcribed by the cellular RNAP and producing ribozymes that cleave a sequence in the *marA* drug resistance gene. The use of optimized single-stranded promoters in combination with synthetic nanocircle DNA vectors represents a potentially useful way to engender the synthesis of biologically active RNAs in living cells.

rolling circle transcription | hammerhead ribozyme | *in vitro* selection

Recent work has shown that very small circular single-stranded DNAs (ssDNAs) can be efficiently transcribed by phage and bacterial RNA polymerases (1–7). Transcription proceeds by a "rolling-circle" mechanism, generating long RNA repeats encoded by the circular DNA. This rolling circle transcription (RCT) has been demonstrated for circles in the size range 18 to ≈110 nt, or roughly 2–10 nanometers in diameter (6, 7), and the long product RNAs have been imaged under the atomic force microscope (8–10). Such DNA circles can be used to encode concatameric or self-cleaving monomeric ribozymes of the hammerhead (2), hairpin (3), and hepatitis delta motifs (4). Although evidence in one case suggests that transcription by an RNA polymerase of unstructured circular ssDNAs can initiate with GTP at any cytosine in a circle (2, 11), it was also observed that secondary structures in circular DNAs can strongly inhibit or enhance transcription in some cases.

It has been known for some time that RNA polymerases can initiate transcription in single-stranded "bubble" regions within duplex DNA (12–14), and in single-stranded DNA homopolymers, particularly in cytosine-rich sequences (15–18). However, to our knowledge, no broad range of sequences or structures have been surveyed for such activity. We sought to investigate whether there are specific sequences and/or structures of single-stranded DNA that would act as promoters for a bacterial RNA polymerase. Here we report the identification of such sequences and structures by an *in vitro* selection strategy. We describe a motif that is formed in only ≈40 nucleotides of contiguous sequence, or considerably smaller than a previously reported naturally occurring single-stranded promoter in *E. coli* (19). It appears to be general, in that it can be transplanted from one context to another and still direct efficient transcription by the bacterial RNA polymerase. We further demonstrate that this promoter can be used in a DNA nanocircle vector (a "rolling circle" vector built from ssDNA) to direct transcription of an active hammerhead ribozyme in *E. coli* cells.

Materials and Methods

Preparation of Oligonucleotides and Circular DNA Library. An initial circular ssDNA library containing 63 nt of fixed sequence and 40 nt of randomized sequence was generated by sequential enzymatic ligations of 5′-phosphorylated 56-nt and 47-nt oligonucleotides by using T4 DNA ligase (New England Biolabs) and 16-nt splint oligonucleotides as described (2). The 5′-phosphorylated sequences were: 5′-pTTC GTC TG-N_{40}-TCT TTC AG-3′ and 5′-TTT CGT CCT CAC GGA CTC ATC AGA ATG GCA ACA CAT TGA CTG AGG AG-3′. Details are given in supporting *Materials and Methods*, which is published as supporting information on the PNAS web site, www.pnas.org.

***In Vitro* Selection.** Conditions for initial RCT reaction were: 1 μM circular DNA, 2 units *E. coli* RNA polymerase holoenzyme (Boehringer Mannheim), 0.5 mM ATP, CTP, and GTP, 60 μM UTP, and 0.30 μCi (1 Ci = 37 GBq) of [α-^{32}P]UTP in 25 mM Tris·HCl (pH 8.1) buffer containing 20 mM NaCl, 15 mM $MgCl_2$, 0.4 mM spermine·HCl, 100 μg/ml acetylated BSA, 10 mM DTT, and 12.5 units/ml RNase inhibitor (Promega), in a total reaction volume of 15 μl. After a 1.5-h incubation at 37°C, the reaction was terminated by adding an equal volume of stop solution (30 mM Na_2EDTA/8 M urea/0.02% bromophenol blue/0.02% xylene cyanol). Self-processed 103-nt product RNAs were purified by 10% denaturing PAGE gels run at 4°C.

After elution from the gels, the selected 103-nt ssRNAs were reverse transcribed (Invitrogen) with a 5′-phosphorylated 18-nt primer (pGAC TGA GGA GTT CGT CTG) for 1 h at 42°C. After buffer exchange with Bio-spin column (Bio-Rad), the cDNA products were PCR amplified with *Taq* polymerase for 15 cycles (temperature cycle: 94°C, 1 min; 55°C, 1 min; 72°C, 1 min) in the presence of 100 pmol each of two primers: 5′-pGAC TGA GGA GTT CGT CTG-3′ and 5′-biotin-AAT GTG TTG CCA TTC TGA-3′. The PCR products were extracted with phenol/$CHCl_3$, ethanol precipitated, and blunted with T4 DNA polymerase (LifeTechnologies, Grand Island, NY).

The blunt-ended PCR products were immobilized on magnetic beads with streptavidin (Dynal, Great Neck, NY) in the presence of 80 μM binding buffer [1 M NaCl/10 mM Tris·HCl

Abbreviations: RT, reverse transcription; CAT, chloramphenicol transferase; RCT, rolling circle transcription; ssDNA, single-stranded DNA.

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(pH7.5)/1 mM EDTA], rinsed with two 80-μl volumes of the binding buffer, and eluted with a 70-μl volume of 0.15 N NaOH to recover the nonbiotinylated ssDNA. The recovered solution was then ion-exchanged with a spin column (Bio-Spin P-30 Column, Bio-Rad).

After quantifying the nonbiotinylated ssDNA with 5′-phosphate by measuring the absorbance at 260 nm, 1 μM nonbiotinylated ssDNA with 5′-phosphate was mixed with 2 μM 24-nt splint DNA (5′-AAC TCC TCA GTC AAT GTG TTG CCA-3′) in the presence of 10 mM Tris·HCl (pH 7.5) and 10 mM $MgCl_2$, annealed with a thermal cycler (rate: 1°C/min) from 90°C to 25°C, and incubated with 0.3 units of T4 DNA ligase, 0.1 mM DTT, and 10 mM ATP at 25°C for 12 h in a final total reaction volume of 15 μl. The circular ssDNA, unreacted ssDNA, and splint ssDNA were ethanol-precipitated and used as templates to begin the next round of *in vitro* selection without purification. The RCT reaction for next round was done under the conditions described above.

Control experiments were carried out as above but eliminating the ligation step. The population without ligation contained 1 μM nonbiotinylated ssDNA with 5′-phosphate and 2 μM 24 nt splint DNA to mimic the selections performed ligation.

Cloning and Sequencing. The PCR products of the fifteenth round pool were ligated into a TA cloning vector (Invitrogen) and cloned into *E. coli* TOP10F′ (Invitrogen). Plasmid DNAs were isolated and sequenced using BigDye terminator cycle sequencing kit (Perkin–Elmer Applied Biosystems).

Synthesis and Activity of Selected Circular ssDNAs. All circular ssDNAs were made by sequential enzymatic ligations as indicated above. Sequences of all circular DNAs are given in supporting *Materials and Methods*.

All RCT reactions were done under condition as indicated above by using *E. coli* RNA polymerase (RNAP) (Boehringer Mannheim) with 0.2 μM circular ssDNA templates. Reactions were incubated at 37°C, and were stopped by the addition of one volume of stop solution. Gel analysis was on a 10% PAGE run at 4°C. The transcribed and self-processed RNAs were quantified with a radioanalytical scanner (Molecular Dynamics Storm 860).

***Mar*A-CAT Chimeric Reporter Construct.** The chloramphenicol transferase (CAT) gene fragment was amplified from pKK232–8 (Amersham Pharmacia) by PCR with 29-nt and 27-nt primers. Primer sequences were: 5′-AGG TCG ACT ATG GAG AAA AAA ATC ACT GG-3′ and 5′-GGT ACC CAA AAG GCC ATC CGT CAG GAT-3′. After purification from an agarose gel, the PCR product was amplified again by PCR with 81-nt and 29-nt primers. The 81-mer primer had 51 nt from the *mar*A gene and a*Hind* III site. The 29-nt primer contained a *Kpn*I site. Primer sequences were: 5′-CCC AAG CTT GTC ACT GGA GAA AGT GTC AGA GCG TTC GGG TTA CTC CAA ATG GCA CCT GCA AAT GGA GAA AAA AAT CAC TGG and 5′-GGG GTA CCC AAA AGG CCA TCC GTC AGG AT. After purification from an agarose gel, this *mar*A-CAT fragment (1300 nt) was ligated into pUC19 (Amersham Pharmacia) at the *Hin*dIII site and *Kpn*I site with T4 DNA ligase. The ligated plasmids were cloned into INVαF′ *E. coli* (Invitrogen) and selected by blue–white colony color.

CAT Assay. The INVαF′ *E. coli* strain (Invitrogen) was used. After cells were transfected with the CAT vector, cells were cultured in LB containing 50 μg/ml ampicillin until the absorbance at 600 nm reached 0.1. Then 1 μl of cell solution with 0.1 absorbance was transferred into 100 μl of LB solution with the circular DNA and incubated until the absorbance at 600 nm became 0.1–0.2. Cells were heat-shocked (42°C) once each hour. The CAT activities were measured with [^{14}C]chloramphenicol (100 μCi/ml) and a CAT enzyme assay system (Promega). The efficiency of CAT protein extraction was checked by reference to β-galactosidase activity: cells were cotransfected with pSV-β-Galactosidase Control Vector (Promega) and then the chemiluminescent signal due to β-galactosidase was determined with β-galactosidase enzyme system (Promega).

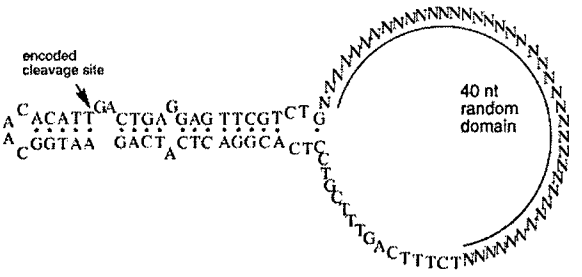

Fig. 1. Sequence of 103-nt single-stranded DNA nanocircle library containing 40 nt of randomized sequence, and 63 nt of fixed sequence encoding a hammerhead ribozyme.

Reverse Transcription (RT)-PCR Assay for Cleavage of *mar*A MRNA. After cells were incubated with or without the circular DNA, when the absorbance at 600 nm became 0.1–0.2, the total RNAs were isolated with the SV Total RNA Isolation System (Promega). The isolated RNAs were reverse transcribed using ThermoScript RT-PCR system (Life Technologies) with a 22-nt primer (5′-GTA TAT CCA GTG ATT TTT TTC T-3′) for 1 h at 65°C. Then the cDNA products were PCR amplified by 25 cycles (temperature cycle: 94°C, 1 min; 55°C, 1 min; 72°C, 1 min) in the presence of two different primer sets. The first primer set: 5′-GTA TAT CCA GTG ATT TTT TTC T-3′ and 5′-ATG ACC ATG ATT ACG CC-3′. The second primer set: 5′-GTA TAT CCA GTG ATT TTT TTC T-3′ and 5′-AGA GCG TTC GGG TTA CTC CA-3′. The PCR products were quantified with NIH IMAGE software.

Results

***In Vitro* Selection Strategy.** To identify single-stranded DNA sequences that enhance or promote transcription, we built a single-stranded library containing a 40-nt randomized domain, incorporated into a 103-nt single-stranded DNA nanocircle (Fig. 1). The initial library in the experiments contained an estimated 10^{13} different single-stranded circular sequences within the same context. A small circular context has the advantage that if a given member of the library were transcribed, the result would be isothermally amplified by a rolling circle mechanism (1). Also encoded in the library nonrandom domain was a 63-nt hammerhead ribozyme and its substrate for cleavage. RCT would be expected to result in synthesis of long repeating RNAs, followed by self-cleavage in the Mg^{2+}-containing buffer (2). The ultimate product of this process would be a monomeric 103-nt ribozyme RNA of strictly defined length.

Our overall scheme for selection involved transcription of the circular library by *E. coli* RNA polymerase holoenzyme, separating the products (after self-processing) on a denaturing gel, excising monomer-length RNAs, amplifying them by RT-PCR, and then religating one strand from the PCR reaction to generate a new circular library enriched in sequences that are transcribed well (details are available in supporting *Materials and Methods*). It is worth noting that this scheme selects for more than merely transcription efficiency: it also tends to select for ribozymes that are efficient at self-processing, for DNA sequences that are efficiently ligated to circular form, and for

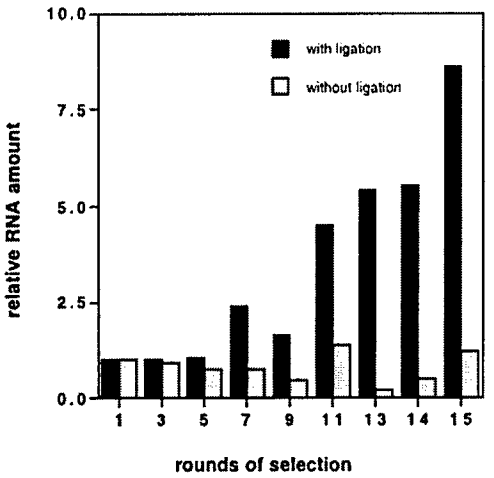

Fig. 2. Improvement of transcription activity over successive rounds of *in vitro* selection. RNA amount was measured for each successive population at 37°C after 1.5 h. Dark and light bars correspond to the relative RNA amounts (>80-nt product) for the successive population with and without ligation, respectively. The reaction conditions are described in *Materials and Methods*.

sequences that are efficient at being amplified by RT-PCR. To encourage optimization of transcription, we kept transcription times relatively short and self-processing and ligation times relatively long.

We carried out fifteen rounds of selection and amplification by this approach. Fitness of the library (measured by total amounts of RNA >80 nt produced) generally increased over the generations (Fig. 2). After fifteen rounds the fitness was increased several-fold over the original randomized set, and did not increase with further rounds (data not shown). Ligation to circular form was necessary for increasing fitness, because the unligated DNAs did not show increasing production of RNA (Fig. 2), consistent with a rolling circle mechanism of transcription. We then cloned the PCR fragments after round 15 and sequenced plasmids from 38 individual colonies. Selected sequences are shown in Fig. 3.

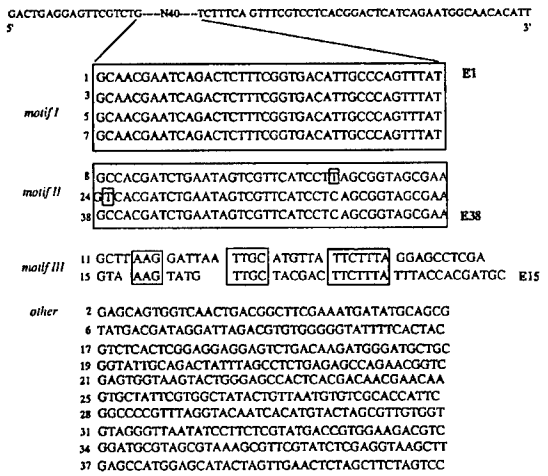

Fig. 3. Sequences of clones obtained following the fifteenth round of *in vitro* selection. Boxes indicate regions of high sequence similarity.

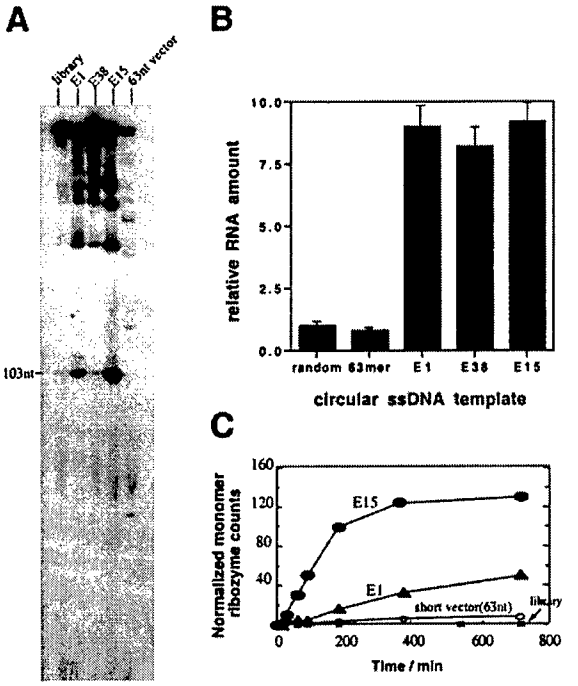

Fig. 4. Selected circular DNA motifs engender RNA synthesis *in vitro* with *E. coli* RNAP. (*A*) Autoradiogram of denaturing 10% polyacrylamide gel showing *in vitro* transcription of the 103-nt initial library, a control 63-nt nanocircle lacking the randomized domain, and selected individual nanocircles E1, E15, and E38 (after 1.5 h). (*B*) The relative total RNA amounts (all lengths >80 nt) for the 103-nt initial library, 63-nt nanocircle lacking the randomized domain, and E1, E15, and E38. (*C*) Time course of the production of monomeric ribozyme for the 103-nt initial library (■), 63-nt nanocircle lacking the randomized domain (○), E1 (▲), and E15 (●).

Selection Yields Strongly Transcribed Motifs in Circular ssDNAs. The results from sequencing revealed three main sequence motifs that were represented more than once, as well as ten sequences represented only once (Fig. 3). The presence of multiple examples of some of the motifs suggests that selection had indeed enriched the population with cases having superior fitness. Overall, there is a small bias toward T-rich sequences (28% T) and toward a deficiency in C (22% C). It is possible that this reflects the known preference of *E. coli* RNA polymerase (RNAP) for initiation with ATP opposite T, although some of the best-transcribed sequences are not T-rich (e.g., E38, see below). Alternatively, it may reflect some nucleotide bias in the initial library.

We evaluated fitness of individual selected motifs by separately examining one member of each of the three most common motifs. The individual DNA circles (labeled E1, E15, and E38 in Fig. 3) were constructed in the same way as the library and were then evaluated for their ability to engender RNA synthesis *in vitro* with *E. coli* RNA polymerase. The results showed (Fig. 4 *A* and *B*) that all three were considerably better transcribed than either the initial randomized nanocircle library, or than a 63-mer nanocircle lacking the randomized domain altogether. In addition to enhancement of total RNA synthesis, the rate of production of monomeric ribozymes was also enhanced for E15 and E1 (Fig. 4*C*), with that for E15 being the greatest. Motif E38 produced RNA that was relatively slow at self-processing (Fig. 4*A*) but produced the most total RNA, being 80-fold more strongly transcribed than the original circular library.

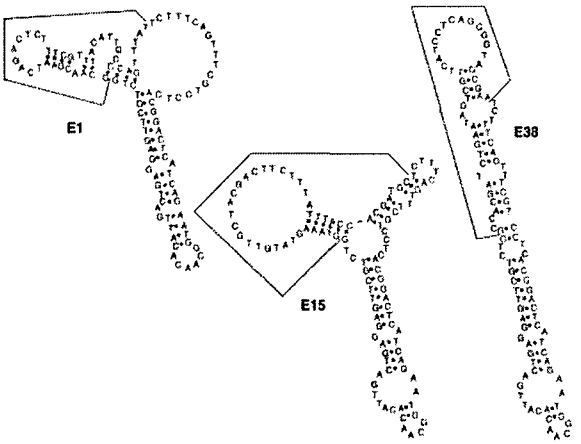

Fig. 5. Sequences and predicted secondary structures of E1, E15, and E38. Boxed part indicates selected sequence from original randomized 40-nt domain; unboxed part encodes ribozyme.

Finally, we asked whether canonical *E. coli* promoters would be active in the same context. We prepared a circle containing the 63-nt ribozyme constant domain along with a 42-nt *E. coli* tac promoter bottom strand (20). Transcription of this circle alone (bottom strand only) or in the presence of an equimolar amount of a 42-nt top strand complement showed that neither was transcribed well in comparison to the E15 sequence (data not shown). Based on amount of monomer ribozyme produced, the E15 circle produced 20-fold more RNA than the case with both strands of the *tac* promoter, and 40-fold more than with the bottom strand of the promoter alone. This finding is consistent with a previous observation that RCT can produce more RNA than DNAs having canonical promoters (1).

We evaluated the predicted secondary structures for the selected cloned circular DNAs (21). There was no apparent structural similarity predicted for the randomized domains, save for the fact that all formed bulged stem–loop structures without exceptionally strong secondary structure. There appeared to be no correlation between predicted free energy of the folds and transcription ability. This finding may reflect the fact that there is likely more than one way to increase the amount of monomer RNA produced. For example, increased initiation efficiency, increased processivity during transcription, increased self-processing ability, and increased ability of the DNA to be ligated to circular form may all contribute to fitness. Predicted structures for E1, E15, and E38 (Fig. 5) are all significantly different (21), which may not be surprising if different winning strategies are involved; however, we have no confirmation of what secondary structures are formed *in vivo*.

As mentioned above, one naturally occurring single-stranded promoter was previously identified that is active in *E. coli* (19). We thought it possible that some of our selected small motifs might also be active, or at least present and therefore potentially active, in *E. coli*. However, a search of the *E. coli* genome showed no significant similarities with our selected motifs.

Transplantation of Promoter Motif. As a further test of transplantability of a selected pseudopromoter motif, we constructed a new nanocircle vector encoding a different hammerhead ribozyme (the sequence is given in supporting *Materials and Methods*). This new ribozyme is targeted to *mar*A RNA, which encodes multiple antibiotic resistance in certain pathogenic strains of *E. coli* (22), and is predicted to cleave it between nucleotides 40 and 41. This new ribozyme (marA103) is different from the previous E15 by

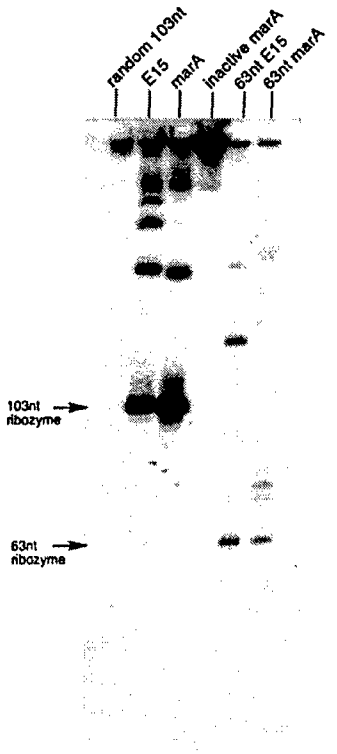

Fig. 6. Assessment of transplantability of E15 selected motif to a new nanocircle encoding marA ribozyme. Autoradiogram of denaturing 10% polyacrylamide gel showing *in vitro* transcription of the 103-nt initial library, nanocircle E15, the new *marA* nanocircle, *marA* nanocircle with inactivated ribozyme, and two 63-nt nanocircle controls.

13 nucleotides in sequence. We measured transcription efficiency of this new 103-nt nanocircle with *E. coli* RNAP, comparing it to the previous E15 vector as well as to a 63-mer circle lacking the selected promoter-like domain (marA63). Results showed (Fig. 6) that it retained full activity as compared with the E15 vector and, indeed, exceeded that activity by a significant factor. Once again, its activity was much higher than the 63-mer lacking the promoter-like sequence. Thus we conclude that at least conservative switches can be made in local structural and sequence context, with full retention of transcriptional activity. Finally, an A → C mutation in the conserved hammerhead domain of marA103 inactivated the cleavage activity of the ribozyme but did not diminish transcription, producing a long concatameric RNA (Fig. 6, 4th lane).

A Selected Nanocircle Vector Is Active Against marA RNA in *E. coli*. We then evaluated whether this marA103 nanocircle vector might be transcribed in bacterial cells. If it were transcribed, one expects that multimeric strings of ribozymes would be produced by RCT, and that these would subsequently self-process. The resulting monomeric ribozymes, or the concatameric precursor, could then potentially cleave *mar*A RNA in *trans* (2, 23). To evaluate this possibility we prepared a reporter plasmid construct encoding a 51-nt segment of *mar*A RNA in the upstream end of the CAT gene (the construct is diagrammed in Fig. 10, which is published as supporting information on the PNAS web site, www.pnas.org). Experiments showed that the extra 17 aa did not abolish CAT activity in the chimeric protein. With this construct,

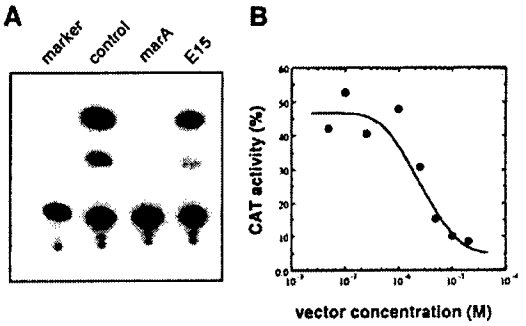

Fig. 7. Effect of nanocircle vectors on the inhibition of CAT activity. (*A*) Thin-layer chromatogram showing levels of CAT expressed in the presence of 10 μM *marA* vector and E15 vector. The control lane is with no nanocircle vector. (*B*) Concentration dependence of down-regulation of CAT activity with *marA* vector.

cleavage of the *mar*A segment of RNA would then be expected to lead to down-regulation of CAT activity by diminishing the amount of translatable CAT mRNA.

We introduced the *mar*A103 nanocircle vector (NCV) and control DNAs into bacteria containing the reporter plasmid by heat shocking the cells. We measured CAT activity (by radiolabeled thin layer chromatography) as a function of nanocircle concentration in the medium during heat shock. Results show that the marA NCV was able to down-regulate CAT activity markedly (Fig. 7*A*) as compared with the control with no vector, and to the case with the E15 vector, which should be transcribed strongly but encodes a non-*mar*A ribozyme (Fig. 8). Varying the amount of the marA103 NCV revealed a concentration-dependent down-regulation of CAT activity (Fig. 7*B*), reaching 50% of maximal activity at *ca.* 7 μM nanocircle concentration and reaching *ca.* 80% inhibition at $\approx$10 μM and above. Control experiments revealed that when a single A $\rightarrow$ C mutation, carried out to inactivate the ribozyme cleavage, was made, activity dropped significantly (Fig. 8), indicating that ribozyme cleavage is important. It also appears that some of the observed down-regulation of CAT may be due to simple antisense activity

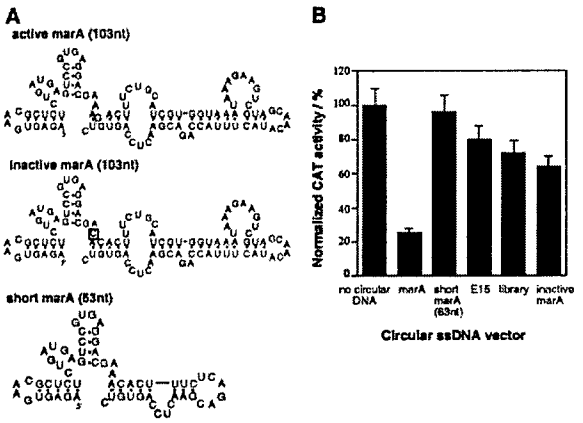

Fig. 8. (*A*) Sequences and predicted secondary structures of the monomer ribozymes: active and inactive *marA*, and short *marA*. The inactivating A $\rightarrow$ C mutation is boxed in the first ribozyme. (*B*) Effect of 10 μM various nanocircle vectors on the inhibition of CAT activity. The plotted data were averaged from three independent experiments.

of the ribozyme RNA, because this inactive marA NCV did show some down-regulation activity. Overall, the data suggest that the main activity of the *mar*A monomeric (or multimeric) ribozymes arises from their ability to cleave the intended target. A separate control was carried out with the 63-mer nanocircle lacking the promoter-like domain (Fig. 8); this circle was inactive over the same concentration range. This result confirms that the greater amounts of RNA generated because of the promoter are needed to show trans-cleavage activity.

Finally, to independently check for cleavage of the intended target RNA in the bacterial cells, we carried out RT-PCR experiments with total isolated RNA, using pairs of primers on either side of the expected cleavage site, comparing the relative amounts of full-length RNA versus the expected shortened fragment [as judged by their corresponding 101- and 60-nt PCR fragments (see Fig. 11, which is published as supporting information on the PNAS web site, www.pnas.org)]. The results show a 96% decrease in full-length RNA in the presence of the marA103 NCV as judged by the drop in amount of 101-nt PCR fragment. Measurement of vector effects on the shorter 60-nt PCR fragment shows that the marA NCV causes a smaller 55% decrease, possibly due to an antisense mechanism. The results are consistent with cleavage between the two limiting primers spanning the intended site, providing further evidence in support of the expected ribozyme-mediated cleavage, decreasing CAT activity.

Discussion

The use of strongly transcribed nanocircle vectors represents a potentially useful way to deliver biologically active RNAs into bacteria. This method is quite distinct from the standard approach of delivering RNAs encoded on plasmids. Nanocircles may be easily used to engender repeating RNAs that would be difficult to encode on a plasmid stably. Although we used such a vector to encode a hammerhead ribozyme, it seems quite possible that other classes of ribozymes, as well as other short RNA motifs (such as antisense RNAs, hairpins, or dsRNAs), may be encoded (3, 4). The nanocircle vectors are not replicated and thus the encoded RNAs are transient in the cell, which may have advantages in certain applications where a drug-like approach to gene inhibition is useful, but disadvantages when permanent transformation is desired. Previous studies have shown that RCT can produce greater amounts of RNA than canonical RNA polymerase promoters, and the present comparisons also support this possibility. If that is the case, then nanocircle vectors may offer a unique way to engender more RNA in a cell than otherwise possible (A. Seyhan, A. M. Diegelman, E.T.K., and J. M. Burke, unpublished observation).

The data allow us to conclude that at least conservative switches can be made in local structural and sequence context, with full retention of transcriptional activity. It remains to be seen whether such a pseudopromoter can be active in transcription of other contexts, such as with other ribozymes or other RNA structural motifs. In cases where transplantability is limited, one could possibly carry out similar selections in those other contexts.

It is worth noting that although the transplanted E15 sequence does promote transcription, it may not necessarily do so by efficiently directing initiation at a specific site. Examination of the transcription products of E15 or the other selected circles shows no strong bands between the self-processed ribozyme bands, which would necessarily exist if transcription were initiated at one site unique from the cleavage site. Thus it is possible that initiation is not rate limiting here, and that selection has generated sequences that are especially good at enhancing processivity rather than initiation. The transcription products seem to suggest that initiation occurs at several sites in the circle. More studies will be required to determine what structural

features, beyond stem–loop and bulged duplexes, are important for strong transcription. Most if not all of the members of the randomized library also contain mismatched (looped) regions, and yet the selected winner is 80-fold more strongly transcribed than the library. It will be of significant interest in future studies to identify how small changes in sequence and structure of some of the selected motifs affect transcription initiation and elongation efficiency. Also of interest would be to examine explicitly the role of the sigma factor in initiation on these sequences; we used a commercial holoenzyme preparation in which presence of sigma subunit was confirmed, although exact stoichiometries were not apparently quantitated.

Previous reports have described optimization by *in vitro* selection of double-stranded naturally occurring promoters for phage and bacterial RNA polymerases (24, 25). The present experiments are distinct from this by involving only single-stranded sequence and using no naturally occurring sequence as the starting point. These new promoter-like motifs are to our knowledge the first cases of new single-stranded promoters for a bacterial polymerase. The *in vitro* selection method we used has the particular advantage that winners are self-amplified by their own RCT, which can potentially lead to rapid gains in population even from rare motifs. We have recently taken advantage of this by selecting for single-stranded promoters for a phage RNA polymerase, complementing the present bacterial polymerase study (Ohmichi and Kool, unpublished work). Future studies will be directed at identifying possible single-stranded promoter motifs for other RNA polymerases.

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